WAVE1 controls neuronal activity-induced mitochondrial distribution in dendritic spines

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Mitochondrial fission and trafficking to dendritic protrusions have been implicated in dendritic spine development. Here, we show that Wiskott–Aldrich syndrome protein (WASP)-family verprolin homologous protein 1 (WAVE1) controls depolarization-induced mitochondrial movement into dendritic spines and filopodia and regulates spine morphogenesis. Depolarization-induced degradation of the p35 regulatory subunit of cyclin-dependent kinase 5 (Cdk5), with the resultant decreased inhibitory phosphorylation on WAVE1, depend on NMDA receptor activation. Thus, WAVE1 dephosphorylation and activation are likely associated with mitochondrial redistribution and spine morphogenesis.

Cdk5 | filopodia | depolarization | phosphorylation | NMDA receptor

Mitochondria play a critical role in neuronal development and synaptic function through their ability to supply ATP and regulate Ca^{2+} homeostasis (1–6). Moreover, mitochondrial abnormalities are associated with many neurological diseases, including neurodegenerative diseases and psychiatric disorders (7, 8). Mitochondria are highly dynamic and undergo constant fusion and fission (9, 10). Mitochondria are transported from the cell body to sites of high-energy utilization through axons and dendrites, and they undergo retrograde transport from the nerve terminal toward the cell body (11). Interestingly, neuronal activity increases the ratio of fission versus fusion and increases the percentage of mitochondria in dendritic spines and filopodia (4). Repetitive depolarization induces the formation of dendritic protrusions (filopodia) (12) and spines (4) in hippocampal neurons, and mitochondria are likely to supply ATP and regulate calcium during spine morphogenesis (4). The presence of mitochondria in spines also might play a role in the regulation of synaptic transmission.

The molecular mechanisms that mediate activity-dependent mitochondrial distribution in dendritic spines are unknown. Interactions between mitochondria and the cytoskeleton are essential for normal mitochondrial motility and distribution (13). Microtubules are the primary means for long-distance mitochondrial transport, whereas the actin cytoskeleton is required for short-distance mitochondrial movements (14). Wiskott–Aldrich syndrome protein (WASP)-family verprolin homologous protein 1 (WAVE1), through its ability to activate the Arp2/3 complex, is a key regulator of actin polymerization and dendritic spine morphology (15). Here we show that WAVE1 is required for activity-dependent mitochondrial trafficking to dendritic spines and filopodia.

Results

Localization of WAVE1 in Mitochondria. Previous studies have shown that WAVE1 is present in dendrites, dendritic spines, and axonal growth cones (16–18). Because both WAVE1 (15) and mitochondria (4) are implicated in dendritic spine formation, we examined the localization of WAVE1 and mitochondria in the dendrites of primary-cultured hippocampal neurons. Mitochondria were visualized after the expression of Mito-DsRed (DsRed2 fused to the mitochondrial targeting sequence from subunit VIII of human cytochrome *c* oxidase) (19). Endogenous WAVE1 was visualized by immunostaining with an anti-WAVE1 antibody. Under unstimulated conditions, we observed partial colocalization of WAVE1 and mitochondria in the dendrites of hippocampal neurons (≈44% of WAVE1 in dendrites was colocalized with mitochondria) (Fig. 1). After neuronal stimulation by using a repetitive depolarization protocol (4, 12), mitochondrial fission occurred (see also Fig. 2 and the associated text) and was accompanied by almost complete colocalization of WAVE1 and mitochondria (≈80% of WAVE1 in dendrites was colocalized with mitochondria).

The colocalization of WAVE1 and mitochondria is consistent with previous reports. WAVE1 was identified in a protein complex with BAD, a proapoptotic Bcl-2 family member from liver mitochondria (20). Immuno-EM of purified mitochondria showed the presence of WAVE1 in the outer membrane of mitochondria (20). In addition, focal ischemic stroke has been found to induce the formation of a protein complex including WAVE1, pancortin-2, and Bcl-xL in mitochondria-enriched fractions from the brain (21). Thus, WAVE1 is likely to be associated with the mitochondrial outer membrane in neuronal and nonneuronal cells.

WAVE1 Mediates Depolarization-Induced Trafficking of Mitochondria to Dendritic Spines. Live-imaging studies have revealed the highly dynamic nature of mitochondrial morphology. The shape, size, and number of mitochondria are regulated through the control of fusion and fission (9, 10). In neurons, previous studies have found that neuronal activity results in increased fission of mitochondria and their movement into dendritic spines and filopodia (4). Based on the results shown in Fig. 1 and results from other studies, WAVE1 appears to interact with the outer mitochondrial membrane. Therefore, WAVE1 might be expected to play a role in activity-dependent mitochondrial movement into dendritic spines. Therefore, we analyzed the effects that the reduced expression of WAVE1 had on mitochondrial redistribution into dendritic protrusions.

Under basal conditions, we found that in hippocampal neurons ≈9% of the mitochondria found in dendrites were observed in dendritic protrusions (including filopodia and spines) and that ≈7% of protrusions contained mitochondria (Fig. 2 *A Top Left*, *B*, and *C*). Repetitive depolarization induced the fission of mitochondria and resulted in movement of mitochondria into dendritic spines and filopodia (Fig. 2 *A Top Right*, *B*, and *C*). These results are similar to those obtained by Li *et al.* (4). Notably, the vast majority of dendritic protrusions (≈88%) lacks mitochondria even after stimulation. The reduced expression of WAVE1, as a result of RNAi (15), resulted in a significant decrease in the number of mitochondria in dendritic protrusions under basal conditions and prevented the effect of repetitive

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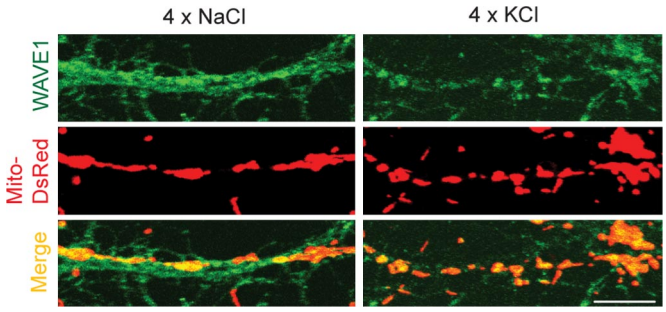

Fig. 1. Localization of WAVE1 in the mitochondria. Primary hippocampal neurons were transfected with Mito-DsRed (DsRed2 fused to the mitochondrial targeting sequence of cytochrome *c* oxidase). Neurons were stimulated with four repeated depolarizations (4× KCl) (50 mM KCl in CM for 3 min, followed by a 10-min interval with CM). NaCl (50 mM) replaced KCl in the controls (4× NaCl). Endogenous WAVE1 was detected with anti-WAVE1 antibody, and Mito-DsRed was detected with anti-DsRed antibody. (Scale bar: 10 μm.)

depolarization (Fig. 2 *A Middle*, *B*, and *C*). Notably, mitochondrial fission in response to depolarization still occurred in RNAi-transfected neurons (Fig. 2*A Middle*). The expression of RNAi-resistant WAVE1 (Rr-WAVE1) in conjunction with RNAi restored basal and activity-dependent mitochondrial movement into dendritic protrusions (Fig. 2*A Bottom*, *B*, and *C*). These results highlight a critical role for WAVE1 in control of mitochondrial trafficking in neurons.

Repetitive Depolarization Induces WAVE1 Dephosphorylation via NMDA Receptor Activation. WAVE1 is phosphorylated by cyclin-dependent kinase 5 (Cdk5) at serine 310, serine 397, and serine 441 in neurons, and the ability of WAVE1 to regulate Arp2/3-dependent actin polymerization is inhibited by phosphorylation (15). In mature neurons in the brain or in culture, WAVE1 is highly phosphorylated at these Cdk5 sites. As a result, WAVE1 is largely inactive under unstimulated conditions (15). Repetitive depolarization reduced the level of phosphorylation at all three Cdk5 sites in WAVE1 in primary cortical neurons (Fig. 3 *C–F*). Depolarization also resulted in the down-regulation of the p35 regulatory subunit of Cdk5 (Fig. 3*A*) while having no effect on Cdk5 level (Fig. 3*B*). Coincubation with the NMDA receptor antagonist 2-amino-5-phosphonovaleric acid (APV) blocked p35 down-regulation and the decrease in WAVE1 phosphorylation, but coincubation with an AMPA/kainate receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) had no effect. Similar results using the NMDA receptor antagonist also were found in hippocampal neurons (Fig. 4). We also observed that bath application of 100 μM NMDA to primary cultured cortical neurons induced the dephosphorylation of WAVE1 and the down-regulation of p35 (data not shown). Thus, repetitive depolarization likely activates WAVE1 through NMDA receptor-mediated down-regulation of p35.

WAVE1 Mediates Repetitive Depolarization-Induced Morphological Changes of Dendritic Spines. Stimulation of NMDA receptors is associated with the outgrowth of filopodia (23) and regulates bidirectional spine morphology during synaptic plasticity: spine formation with long-term potentiation (LTP) induction but retraction with long-term depression (LTD) (24–26). Repetitive depolarization resulted in NMDA receptor-dependent protrusion of filopodia from dendritic shafts, as well as filopodial extension from existing spines (12). We observed filopodial outgrowth from dendritic shafts after repetitive depolarization (Fig. 5*A*). In addition, repetitive depolarization induced bifurcated or forked (multiheaded) spines that were likely to result from filopodial extension from existing spines (Fig. 5 *A* and *B*) (12). The down-regulation of WAVE1 by using RNAi abolished

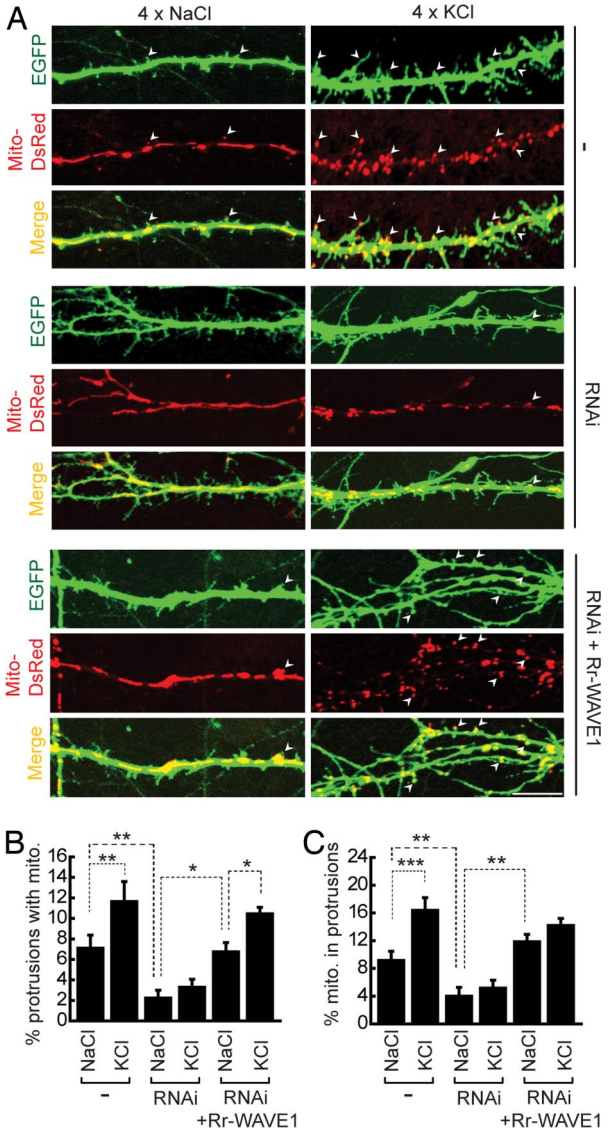

Fig. 2. WAVE1 controls depolarization-induced trafficking of the mitochondria to dendritic spines. (*A*) Hippocampal neurons were cotransfected with EGFP and Mito-DsRed plus control plasmid vectors (−), WAVE1 RNAi (RNAi), or WAVE1 RNAi plus RNAi-resistant WAVE1 (Rr-WAVE1). Neurons were stimulated with NaCl or KCl as in Fig. 1 (4× KCl or 4× NaCl). Arrowheads indicate the mitochondria in spine heads. (Scale bar: 10 μm.) (*B* and *C*) Quantification of percentage of dendritic protrusions (spines) containing mitochondria (*B*) and the percentage of dendritic mitochondria in dendritic protrusions (*C*). The depolarization protocol increased the number of filopodia while reducing the number of spines. Thus, the total number of protrusions was not significantly affected by the depolarization protocol. Mitochondria were equally distributed in filopodia and spines. Data represent means ± SEM (n = 10–19 dendrites, 3–5 neurons per culture, three independent cultures). Multiple comparison by two-way ANOVA with Bonferroni's posttest. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

depolarization-induced multiheaded spines, an effect that was restored by the expression of RNAi-resistant WAVE1 in conjunction with RNAi (Fig. 5*B*). Wu *et al.* (12) found that MAPK activation was important for repetitive depolarization-induced filopodia formation, but a connection of the MAPK pathway to WAVE1 is not known at the present time.

Discussion

The present results highlight a role for WAVE1 in the control of mitochondrial trafficking to spines and filopodia in neuronal den-

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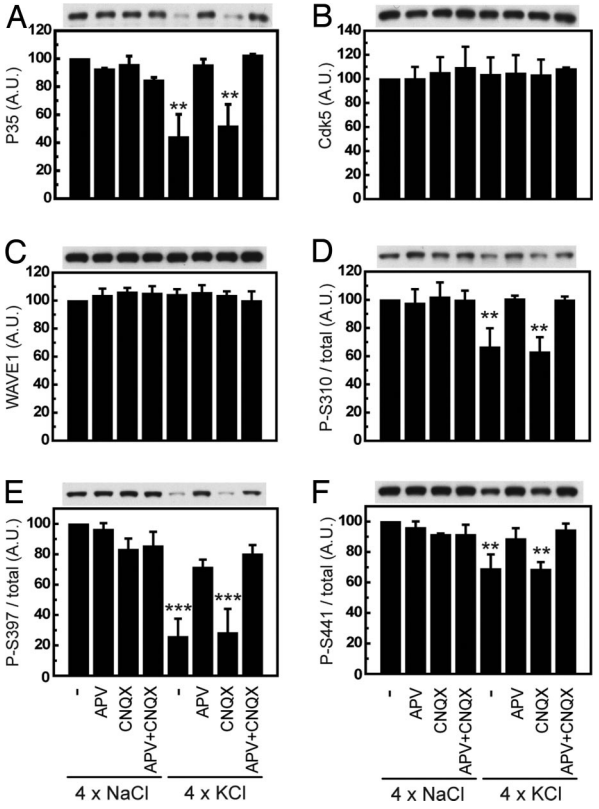

Fig. 3. Depolarization induces WAVE1 dephosphorylation via NMDA receptor activation in primary cortical neurons. Primary cortical neurons were stimulated with 4× NaCl or 4× KCl in the absence or presence of 100 μM APV (NMDA receptor antagonist) and/or 20 μM CNQX (AMPA/kainate receptor antagonist). Levels of total p35 (*A*), Cdk5 (*B*), and WAVE1 (*C*) and the phosphorylation of WAVE1 at P-S310 (*D*), P-S397 (*E*), and P-S441 (*F*) were measured by immunoblotting (*Upper*), and the results were quantified by densitometry (*Lower*). Data that were normalized to the value of 4× NaCl in the absence of antagonists represent means ± SEM for four experiments. **, $P < 0.01$;***, $P < 0.001$ versus the value for 4× NaCl in the absence of antagonists (one-way ANOVA with Neuman–Keuls posttest).

drites. WAVE1 has previously been found to associate with the outer mitochondrial membrane (20), and the actin cytoskeleton has been found to be required for short-distance mitochondrial movement (14). Therefore, WAVE1-dependent movement of the mitochondria to dendritic protrusions is likely to be mediated by Arp2/3-dependent actin polymerization. Whether WAVE1–Arp2/3 complex-mediated actin polymerization drives mitochondrial translocation and/or immobilizes mitochondria in dendritic protrusions is not yet clear. Actin polymerization drives cellular movements, including extension of the leading edge of motile cells, movement of pathogens in the cytoplasm of infected host cells, and movement of organelles such as endosomes and lysozomes (37, 40, 41). In contrast, a functional actin cytoskeleton also has been found to be essential for NGF-induced inhibition of mitochondrial motility and immobilization at sites of NGF stimulation (42, 43).

In mammalian cells, mitochondrial fusion is regulated by mitofusins (29) and OPA1 (30, 31). In contrast, dynamin-related protein1 (Drp1) (32) and Fis1 (33, 34) control mitochondrial fission. Drp1 translocates to the mitochondria and forms a complex with Fis1 (and possibly other proteins) on the mitochondrial surface, where Drp1's GTPase activity is coupled to outer-membrane scission (34, 35). Previous results have shown that the disruption of the filamentous actin (F-actin) cytoskeleton attenuated fission and recruitment of Drp1 to the mitochondria (39). However, our results indicate that depolarization-induced fission still occurred when WAVE1 expression was reduced. Thus, WAVE1 appears to influence the redistribution of smaller mitochondrial species into dendritic protrusions. Fission is presumably required to reduce mitochondrial size before their trafficking into small-sized dendritic protrusions. However, mitochondrial fission and trafficking may be coordinated. During membrane endocytosis, Bin-Amphiphysin-Rvs (BAR)-containing proteins coordinate the actions of dynamin and the actin polymerizing, WAVE1-related protein,

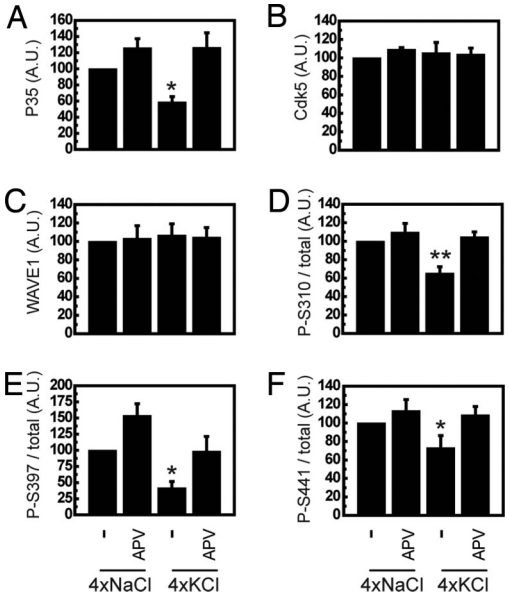

Fig. 4. Depolarization induces WAVE1 dephosphorylation through NMDA receptor activation in primary hippocampal neurons. Primary cultured hippocampal neurons were stimulated with four repeated NaCl (4× NaCl) or KCl (4× KCl) treatments in the absence or presence of 100 μM APV (NMDA receptor antagonist). Levels of total p35 (*A*), Cdk5 (*B*), and WAVE1 (*C*) and the phosphorylation of WAVE1 at P-S310 (*D*), P-S397 (*E*), and P-S441 (*F*) were measured by immunoblotting, and the results were quantified by densitometry. Data normalized to the value of 4× NaCl in the absence of antagonist represent means ± SEM for four experiments. *, $P < 0.05$; **, $P < 0.01$ versus the value of 4× NaCl in the absence of antagonist (one-way ANOVA with Neuman–Keuls posttest).

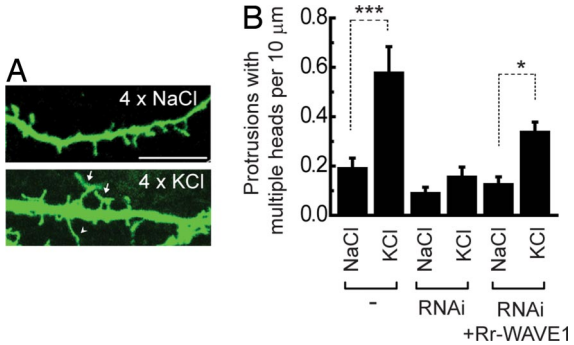

Fig. 5. WAVE1 controls depolarization-induced spine growth. Primary hippocampal neurons were cotransfected with EGFP plus control plasmid vector, WAVE1 RNAi plasmid (RNAi), or WAVE1 RNAi plus RNAi-resistant WAVE1 (Rr-WAVE1). Neurons were stimulated with NaCl or KCl as in Fig. 1. (*A*) Representative images of neurons cotransfected with EGFP plus control plasmid vector showing repetitive depolarization-induced generation of filopodia and multiheaded protrusions. Arrowhead, filopodia; arrows, multiheaded protrusions. (Scale bar: 10 μm.) (*B*) Quantification of protrusions with multiple heads (number per 10-μm dendritic length; means ± SEM, n = 10–17 dendrites, 3–5 neurons per culture, three independent cultures). *, $P < 0.05$; ***, $P < 0.001$ versus control (4 × NaCl) (two-way ANOVA with Bonferroni's posttest).

N-WASP (36, 37). Endophilin B1 is a BAR domain-containing protein that interacts with mitochondria and plays a role in fission (38). However, there have been no reports of any physical interaction of endophilin B1 with WAVE1 and/or Drp1.

WAVE1 is phosphorylated by Cdk5 at multiple sites, and the ability of WAVE1 to regulate Arp2/3-dependent activity is inhibited by phosphorylation in an *in vitro* actin polymerization assay (15). In mature neurons in mouse brain or in dissociated neuronal culture, WAVE1 is highly phosphorylated at these Cdk5 sites. Thus, under basal conditions, WAVE1 is likely to be largely inactive. In support of this supposition, the overexpression of a dephosphorylation-mimicking form of WAVE1 (Ser310Ala), but not of a phosphorylation-mimicking form (Ser310Asp), was able to reverse the morphological defects found after knockdown of WAVE1 expression in neurons (15). In our current studies, we have found that repetitive depolarization resulted in the dephosphorylation of three sites in WAVE1, and this is likely to be necessary to activate WAVE1 and allow mitochondrial redistribution to dendritic protrusions. The effect of repetitive depolarization was dependent on NMDA receptor activation and presumably on an increase in intracellular calcium. We also found that the repetitive depolarization protocol used resulted in the down-regulation of p35. These results are in general agreement with previous studies showing that glutamate treatment of neurons results in the down-regulation of p35 and a decrease in Cdk5 activity (22). The exact mechanism of glutamate-induced degradation of p35 is not known, but is likely to involve the proteasome/ubiquitination system (22) and possible activation of calpain (45). Our preliminary studies found that incubation with the proteasome inhibitor (10 μM MG132) only partially blocked ($\approx$42%) the effect of repetitive depolarization on the degradation of p35 (data not shown). In addition, a small, but significant, amount of p25 was generated after repetitive depolarization ($\approx$30% of that of p35 in the control sample) (data not shown). Thus, in addition to the proteasomal pathway, calpain-mediated p25 generation is likely an additional pathway underlying p35 degradation. Although *in vitro* p25 is able to activate Cdk5, it is believed that an intact NH_2 terminus is needed in p35 to allow proper targeting to the plasma membrane. In p25/Cdk5, the loss of the NH_2 terminus results in the mislocalization and alteration in substrate specificity (49). Thus, our results suggest that, although a small amount of p25/Cdk5 may be formed after repetitive depolarization, this does not efficiently support WAVE1 phosphorylation.

Although degradation of p35 is the most likely candidate involved in decreased WAVE1 phosphorylation, it is also possible that activation of WAVE1 phosphatases plays a role. Our previous studies have found that dopamine-dependent, cAMP-dependent signaling leads to WAVE1 dephosphorylation without any alteration in Cdk5 activity (15). The activation of protein phosphatase(s) is likely to be involved in the actions of cAMP because this was blocked by okadaic acid, a potent inhibitor of protein phosphatase 2A (PP2A) and protein phosphatase 1 (I. Ceglia and Y.K., unpublished data). We have recently shown that a specific heterotrimeric form of PP2A that contains the B56δ subunit can be activated by phosphorylation by protein kinase A (27). Therefore, this mechanism may be involved in dopamine- and cAMP-induced WAVE1 dephosphorylation. We also have shown recently that NMDA receptors can activate a distinct heterotrimeric form of PP2A that contains the B$''$/PR72 subunit (28). Thus, in addition to a reduction of Cdk5 activity through p35 degradation, repetitive depolarization may increase PP2A activity, resulting in WAVE1 dephosphorylation. Whether cAMP signaling also can lead to the redistribution of mitochondria to dendritic protrusions remains to be examined. It also will be of interest to examine the combined effects of glutamate and dopamine because this may have a synergistic action in terms of WAVE1 dephosphorylation and possibly mitochondrial redistribution.

Our results and those of Li *et al.* (4) indicate that neuronal activation results in mitochondrial fission and redistribution of mitochondria into dendritic filopodia and spines. The repetitive depolarization used appears to induce filopodial formation at earlier time points, but spine formation and synaptogenesis at later time points. In our studies, filopodial outgrowth from dendritic shafts and bifurcated or forked (multiheaded) spines, which were likely to result from filopodial extension from existing spines, were observed immediately after repetitive depolarization (see also ref. 12). In the study from Li *et al.* (4), synapse number as measured by PSD-95-positive puncta was increased 9 h after repetitive depolarization. It seems likely that the movement of mitochondria into dendritic filopodia plays a role in their maturation into spines through the provision of ATP or through their ability to buffer local changes in calcium signaling. This hypothesis is supported by the results from Li *et al*. (4), showing that the overexpression of dominant-negative Drp1 or wild-type OPA1 decreased the density of mitochondria in dendrites and decreased the density of dendritic spines. Conversely, the overexpression of wild-type Drp1 increased the density of mitochondria in dendrites and the density of spines. Furthermore, the stimulation of mitochondrial respiration by creatine increased the density of synapses as indicated by PSD-95 puncta (4). However, the establishment of a causal relationship between mitochondrial trafficking into dendritic filopodia and spine maturation remains to be established.

Although the results obtained indicate that WAVE1 is required for the redistribution of mitochondria into dendritic protrusions, only a small percentage of either filopodia or spines were found to contain mitochondria despite the fact that WAVE1 is likely to be distributed widely in spines and filopodia. Mitochondria are rarely found in dendritic spines *in vivo*, as demonstrated by EM analysis of cortical or hippocampal samples (46–48). Therefore, it is likely that mitochondria move into spines only for a relatively short time, and additional live-imaging studies will be necessary to examine how WAVE1 influences mitochondrial dynamics in dendrites, filopodia, and spines. It is clear from our study, as well as from that of Li *et al.* (4), that mitochondria also are found in mature-type spines after neuronal activation. The presence of mitochondria in more mature spines suggests additional functional roles for mitochondria in synaptic transmission or synaptic plasticity. Depolarization-induced spine growth (12), mitochondrial redistribution to dendritic spines (4), and WAVE1 activation are all dependent on the activation of NMDA receptors. The stimulation of NMDA receptors is associated with filopodia formation (23) in developing neurons and regulates spine morphology in different ways. For example, spine size can increase in association with LTP, but decrease in size in association with LTD (24–26). Mitochondria may play specific roles in these various processes.

Although our studies clearly identified a role for WAVE1 in mitochondrial movement into dendritic protrusions, it is likely that WAVE1-dependent actin polymerization plays additional roles in synaptic function. WAVE1 is likely to control actin polymerization and branching in the heads of spines during spine morphogenesis and to play a role in influencing the changes in synaptic morphology that are associated with changes in synaptic plasticity. However, it is also possible that the role that WAVE1 plays in regulating mitochondrial movement impacts the role that WAVE1 plays in regulating actin polymerization in the heads of spines. A greater understanding of the molecular mechanisms, whereby WAVE1 controls mitochondrial movement, should help us to address the precise contribution of WAVE1-mediated mitochondrial distribution to synaptic function.

Materials and Methods

Primary Neuronal Culture and Transfection. Primary hippocampal or cortical cultures were prepared from the brains of embryonic days 17–18 Sprague–

Dawley rats (Charles River Laboratories). After trituration of hippocampal or cortical sections with a glass pipette, for image analysis 1×10^5 hippocampal neurons were plated on a 12-mm-diameter coverslip precoated with poly-L-lysine (BD Biocoat). For biochemical analysis, primary hippocampal or cortical cells were plated in 35-mm dishes precoated with poly-L-lysine (3×10^6 cortical or 1×10^6 hippocampal neurons per dish). Neurons were grown in neurobasal medium supplemented with 0.5 mM L-glutamine, 2% B27, and 1% N2. Fluorodeoxyuridine (10 μM, Sigma) was added to inhibit the proliferation of nonneuronal cells; 50% of conditioned medium (CM) was replaced with fresh medium on 2, 3, 6, and 10 days *in vitro* (DIV). For the analysis of mitochondria and dendritic spines, hippocampal neurons were transfected at 8 DIV by using a calcium phosphate transfection kit (Invitrogen) and were fixed at 12 DIV for immunocytochemistry with anti-DsRed (Clontech), anti-GFP (Abcam), and/or anti-WAVE1 antibodies (44). The pENTR/U6 vector was used for WAVE1 RNAi (Invitrogen). The target sequence of the RNAi is mouse WAVE1 5′-AACGATGAGAAAGGCTTTCCG-3′ (nucleotides 285–305) (15, 50). The empty vector was used as a control. Human WAVE1 was rendered resistant to mouse RNAi by introducing two mutations at positions 300 and 303 (coding for the same amino acid) through two rounds of PCR (15). A significant reduction of WAVE1 was observed after the expression of WAVE1 RNAi (15). In addition, the overexpression of the RNAi-resistant form of WAVE1 in conjunction with RNAi reversed the defective morphology of dendritic spines, supporting the specificity of WAVE1 RNAi (15).

Image Analysis. Fluorescence images were acquired with an LSM510 confocal microscope system (Zeiss) by using a 40× objective lens. A single *z* plane of image was acquired and analyzed. All morphometric measurements were made with Metamorph image analysis software (Universal Imaging). Quantification of the colocalization of WAVE1 and mitochondria in dendrites was performed by using MATLAB (The MathWorks). The intensity of pixels of WAVE1 (green) that overlapped mitochondrial (red) pixels was divided by the intensity of pixels of total WAVE1 (green). For mitochondrial counting, all dendritic protrusions were analyzed. At least two protrusions from a single neck of spines (<3 μm in length) were considered as protrusions with multiple heads. In the case of transfection with WAVE1 RNAi, neurons expressing EGFP were regarded to be cotransfected with the RNAi.

Biochemical Experiments. For biochemical analysis, cortical neurons were treated with KCl or NaCl at 7–8 DIV, and hippocampal neurons were treated at 12–13 DIV. Cells were lysed and sonicated in lysis buffer [50 mM Tris·HCl (pH 7.5), 2 mM $MgCl_2$, 150 mM NaCl, 1% Triton X-100, protease inhibitor mixture (Roche), 1 μM sodium vanadate, 30 mM sodium pyrophosphate, and 30 mM sodium fluoride]. The protein concentration in each sample was determined by the Bradford method. An equal amount of protein was separated by SDS/PAGE, followed by immunoblotting with anti-Cdk5 (C-8) and p35 (C-19) (Santa Cruz Biotechnology), total WAVE1 (anti-C-terminal WAVE1 polyclonal), and phosphorylation state-specific WAVE1 antibodies (15).

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